American Journal of Hematology 58:177–182 (1998)

Phase Changes in Membrane Lipids in Sickle Red Cell Shed-Vesicles and Sickle Red Cells

Maxwell P. Westerman,[1,2*] Lillie Unger,[1,2] Omer Kucuk,[1,2] Peter Quinn,[3] and Leonard J. Lis[1,2]

[1]Division of Hematology/Oncology, University of Health Sciences/The Chicago Medical School, North Chicago, Illinois
[2]Mt. Sinai Hospital Medical Center, Chicago, Illinois
[3]Department of Biochemistry, King's College London, Campden Hill, London, United Kingdom

Lipid phase transformations may occur in the membranes of sickle red cell shed-vesicles and sickle red cells. The presence of such phase changes could be important in sickle cell disease since membrane phase changes appear to contribute to the generation of antiphospholipid antibodies that are thrombophilic and occur in sickle cell disease. In the present study, we have evaluated sickle red cell shed-vesicles and sickle red cells for the presence of non-bilayer lipid phases using ^{31}P-NMR spectroscopy. Results show that the spectra of both the shed-vesicles and the sickle red cells are compatible with the occurrence of non-bilayer phases in the membrane bilayers. The findings support the concept that these membranes could contribute to the generation of antiphospholipid antibodies in sickle cell disease. Am. J. Hematol. 58:177–182, 1998. © 1998 Wiley-Liss, Inc.

Key words: sickle red cell vesicles; membrane phase changes; antiphospholipid antibodies

INTRODUCTION

Although the membranes of red blood cells are in a bilayer lipid phase[a], localized transformations to non-bilayer phases might readily occur, particularly in vesicles shed from sickle red cells (RBC) and in sickle red cells. The vesicles have alterations in membrane phospholipid organization [1–3] and lack certain cytoskeletal polypeptides [4] that could contribute to the formation of non-bilayer phases while vesicle formation is associated with membrane fusion and loss of lipid asymmetry [5,6], which could further affect membrane phases. Sickle red cells might similarly show non-bilayer lipid phase changes due to an increased influx and accumulation of Ca^{2+} in the cells [7–9], increased oxysterol content in the cells [10–12], and reorganization of membrane lipid distribution [13].

Such phase transformations in the sickle red cell shed-vesicles and in the sickle red cells could be important since phase changes appear to contribute to the generation of antiphospholipid antibodies [14] that are thrombophilic and are found in patients with sickle cell disease [15,16]. The antibodies could, thus, contribute to the clotting abnormalities observed in the disease [17]. The presence of a non-bilayer lipid phase also appears to be necessary for the reaction of antiphospholipid antibodies against bilayer lipid phase phospholipids such as phosphatidylserine [18].

Since these phase transformations in the shed-vesicles and in the red cells could contribute to antiphospholipid antibody formation and potentially to clotting abnormalities in sickle cell disease, we have examined the shed-vesicles and the red cells for the presence of non-bilayer lipid phases using ^{31}P-NMR spectroscopy.

METHODS

Patients with sickle cell anemia and normal controls were studied. Blood for vesicle analysis was obtained from 11 patients. None of the patients had received trans-

*Correspondence to: M.P. Westerman, M.D., Hematology/Oncology Unit, Mt. Sinai Hospital, 15th and California Avenues, Chicago, IL 60608.

[a]The term, phase, usually refers to an ordered three-dimensional array of atoms, molecules, or organic structures. In this paper, phase can also refer to an unordered array of structures, or even to individual structures.

Received for publication 18 April 1997; Accepted 18 March 1998

fusions during the year prior to the removal of blood. The total amounts of blood taken from each patient varied between 100–200 ml. Microvesicles were prepared as previously described [4]. Blood was collected with heparin, EDTA, or citrate as the anti-coagulant and immediately prepared for vesicle preparation. No difference was observed between the anti-coagulants used. The blood was centrifuged at 2,500 rpm for 5 min, the supernatant discarded, and the cells washed 4 times with 0.15M NaCl containing 10 mM HEPES-NaOH buffer pH 7.4. The buffy coat was removed. The cells were resuspended in the same medium to a 10% suspension and deoxygenated by incubation at 37°C under an N_2 atmosphere for 1 h. The cell suspension was then centrifuged at 1,500 rpm for 5 min and, after removal of the supernatant, was resuspended in the same volume of the original medium. Reoxygenation and restoration of the original discoid shape of the cells was achieved by exposure to air for 30 min at 37°C. The suspension was again centrifuged at 1,500 rpm for 5 min. The resulting supernatant was removed and centrifuged at 16,000 rpm for 10 min, giving a small red pellet. Cell lysis after reoxygenation was <1%. The red pellet was resuspended in 8 ml HEPES buffered saline, centrifuged at 1,500 rpm for 5 min to remove any contaminating cells, the supernatant removed and centrifuged again at 16,000 rpm for 10 min. The final red pellet was resuspended in 0.5 ml HEPES buffered saline and used for spectroscopic examination. Adequate specimens for spectroscopic examination were obtained from the combined pellets prepared from 170–200 ml packed RBC. The combined pellets were stored at 0°C, which did not alter spectra, and were examined within 7 days. The final combined pellets used for analyses were obtained from 2 groups of patients.

For preparation of red cell membranes, 25 ml of blood was collected with heparin, EDTA, or citrate as the anticoagulant and centrifuged at 2,500 *g*. No difference was observed between the anticoagulants used. The plasma was removed and the red cells washed three times in phosphate buffered saline (0.15 M NaCl, 0.001 M PO_4, pH 7.5). The supernatant and buffy coat were removed. Samples were spun for 5 min at 2,500 *g* after each wash. Hypotonic phosphate buffer (pH 8.0) (10 volumes) was added to the pellet to lyse the red cells and the sample centrifuged at 18,000 rpm for 15 min in a Sorvall (Wilmington, DE) RC-5C with a SS 34 fixed angle rotor. The supernatant was discarded and the pellet was washed 5–7 times in the same buffer until it became light pink in appearance. Two or more washes were done in Hepes buffer to minimize the presence of residual phosphate ions. Two samples of sickle red cell membrane preparations and two samples of normal red cell membrane preparations were examined. Spectroscopic examination has previously been shown to be sensitive to the presence of non-bilayer phases in RBC membranes [19].

A Bruker CXP-180 NMR spectrometer (Wissembourg, France) equipped with a 20 mm ^{31}P probe was used for spectral determinations. 72 MHZ ^{31}P-NMR spectra were obtained at 25°C without proton decoupling using a 450 resonance frequency pulse and an 800 millisecond interpulse time. For sickle RBC shed-vesicles, up to 100,000 acquisitions were obtained. For sickle cell ghosts and normal ghosts, up to 7,000 acquisitions were obtained. Two spectra from the different samples were examined at 21°C by ^{31}P-NMR analyses. Spectral differences did not occur in specimens stored at −20°C for 7 days as compared to freshly obtained samples.

RESULTS

The powder patterns of the spectra from ^{31}P-NMR spectroscopy reflect the symmetry of the corresponding lipid phases (Fig. 1). Phospholipids in a bilayer phase have axial symmetry that gives rise to broad asymmetric spectra in the high field region with a broad low field shoulder (Fig. 1a). Hexagonal phospholipid phases consisting of a two-dimensional ordering of lipid cylindrical structures also have axial symmetry but with a different chemical shift anisotropy, which causes narrower spectra in the low field region with a high field shoulder (Fig. 1b). Other lipid phases, e.g., micellar structures, produce centered symmetric spectra due to rapid isotropic rotation (Fig. 1c) [19].

The sickle red cell shed-vesicles show the presence of non-bilayer phases. A representative spectrum from the vesicles, which are unilamellar membrane bound capsules of hemoglobin with a diameter of approximately 150 nm [20,21] and a lipid composition similar to sickle and normal RBC [4,21], is shown in Figure 2. The spectrum has a single relatively narrow peak in the center of the low field region of the spectrum at 3.8 ppm from 0.

Representative spectra of sickle and normal RBC membranes and the resulting difference spectrum are shown in Figure 3. The spectrum for normal RBC membranes (Fig. 3a) shows a broad asymmetric peak in the high field region and a broad low field shoulder, compatible with a bilayer phase with a superimposed isotropic peak. The isotropic peak (3.0 ppm from 0) would be due to free phosphate ions remaining in the ghost interior compartment after sample preparation. The sickle RBC spectrum (Fig. 3b) is similar to the normal RBC spectrum. However, it contains a broadening of the low field contribution due to the presence of non-bilayer phase structures, i.e., micellar or hexagonal, as well as the superimposed isotropic peak due to the remaining free phosphate ions.

The spectra of the sickle and normal cell membranes are enhanced by the difference spectrum (Fig. 3c) obtained by a point by point subtraction of the intensity of a normal membrane spectral point (b) from the intensity

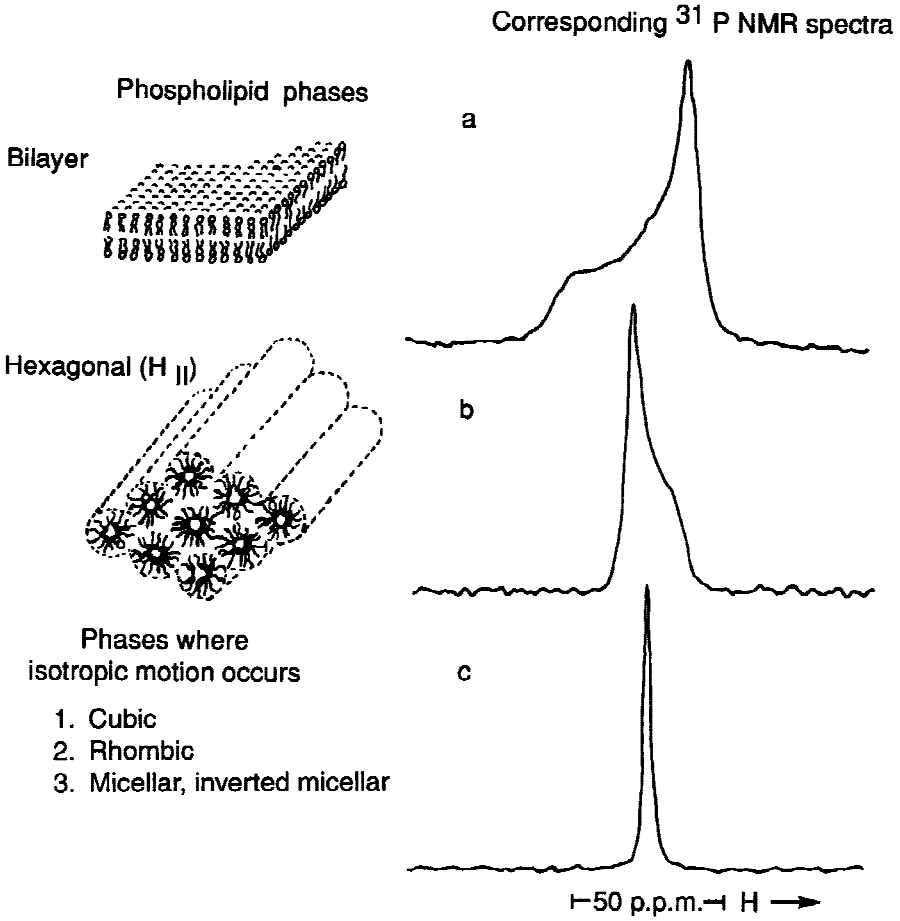

Fig. 1. Diagramatic representations of phospholipid phases and corresponding ^{31}P-NMR spectra. a: Bilayer phase. b: Hexagonal$_{II}$ phase. c: Phases where isotropic motion occurs. Modified from Cullis and Hope [19] with permission from *Nature,* Copyright 1978, Macmillan Magazines Limited.

of an equivalent point in the sickle membrane spectrum (a) using the formula [(b) − 1.31(a)]. Analysis of the difference spectrum is necessary since the membrane remains intact and only a limited population of cells may show changes [22,23]. The non-bilayer phases in the membranes most likely occur within domains, rather than throughout the membrane, in order to maintain membrane structural integrity.

The difference data show a residual positive low field contribution and a residual negative high field contribution. This indicates the presence of a residual spectral contribution of a low field non-bilayer phase peak, due to localized transformation to non-bilayer phase structures in the sickle RBC membrane, and a residual spectral contribution of a high field bilayer phase peak due to the remaining membrane bilayer structures. Although the difference data have a low signal to noise, the results are consistent with having non-bilayer lipid phases in the sickle red cell bilayer membrane.

The low signal to noise, which was observed in the spectra of the RBC membranes, would be expected. It is unlikely the non-bilayer phases, composed of either hexagonal or micellar lipid phase structures, would produce a three-dimensional array in biological membranes due to the inherent two-dimensional order of the intact membrane bilayer structure and the relatively small amount (2–3%) of non-bilayer phase forming lipid present in the membrane [2,4]. Thus, any change in the membrane ^{31}P-NMR spectra, due to the presence of unorganized low concentration non-bilayer phases, would be weak, resulting in a relatively low signal to noise ratio as compared to that observed in a highly organized, very concentrated three-dimensional, pure lipid non-bilayer phase.

DISCUSSION

The present study shows that the membranes of the shed microvesicles and sickle RBC membranes have spectral features consistent with an increase in the amount of non-bilayer phase within the bilayer membrane. These non-bilayer phases would be contiguous with the bilayer lipid phase of the biological membrane [9]. The findings are compatible with the concept that the antiphospholipid antibodies observed in sickle cell anemia [15,16] may be generated from non-bilayer phases [14] in the circulating microvesicles shed from sickle RBC and/or in the sickle RBC membranes. The formation of antiphospholipid antibodies, which are thrombophilic, could contribute to the accelerated clotting activ-

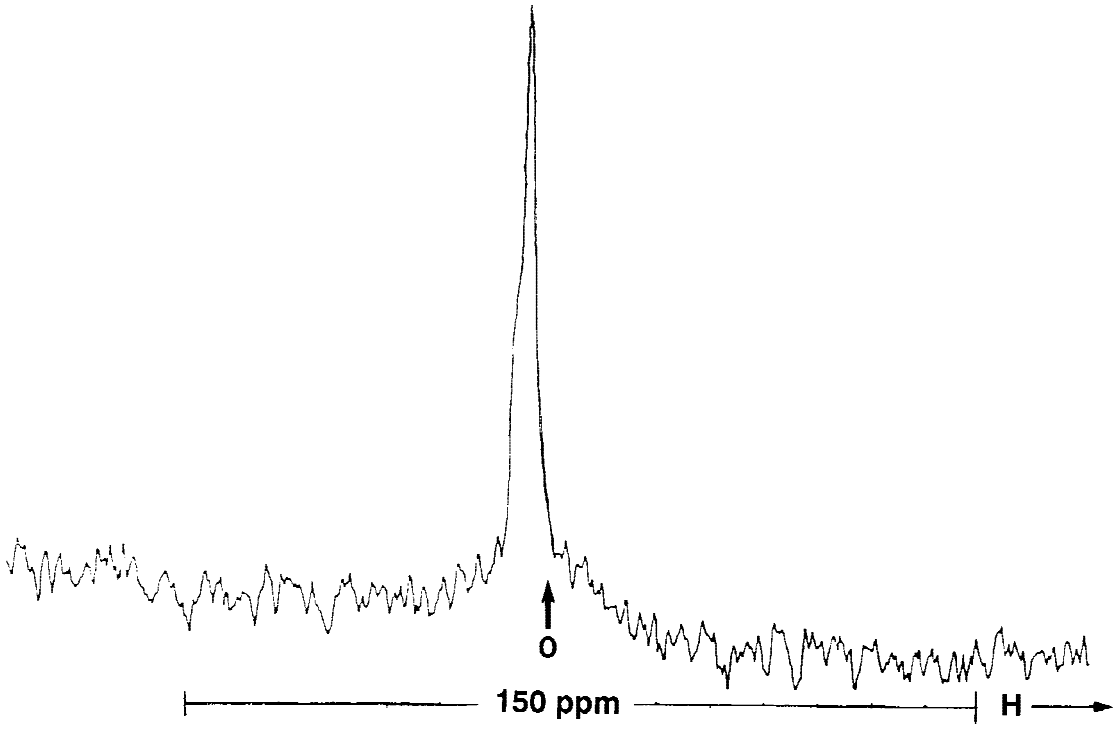

Fig. 2. Representative ^{31}P-NMR spectrum of microvesicles shed from sickle red cells.

ity observed in sickle cell anemia [17]. It is of interest that the vesicles, per se, have a much stronger coagulant effect than sickled red cells [1,2,21].

The spectra obtained from the shed microvesicles of the sickle RBC were unlike those of any other systems observed and are different from those of intact red cell membranes (Fig. 3b). The peak in the spectrum reflects the presence of extensive non-bilayer phase regions within the vesicle lipid bilayer and/or a lipid ''phase'' formed by the association of the lipid vesicles, indicating the presence of vesicle surface interactions resulting in some lipid vesicle ordering [24]. The lack of certain cytoskeletal polypeptides, i.e., spectrin, ankyrin, band 4.1, and actin [4], within the shed vesicles combined with an extensive scrambling of the lipid concentration between bilayer leaflets, could influence the surface interaction between vesicles, allowing for short range spatial ordering of the vesicles with each other. The ordering of shed vesicles would require a change in the surface composition of the vesicles relative to the cell membranes. These vesicles would thus have interactive surface forces that could influence the production of antiphospholipid antibodies.

The peak (3.8 ppm from 0) in the vesicle spectrum (Fig. 2) differs from the free phosphate peak (3.0 ppm from 0) and is too broad to be assigned to an isotropic signal from free phosphate in the buffer. It is also not likely that the spectra of the vesicles was caused by smaller vesicles shed by the sickle RBC, i.e., nanovesicles [25,26], which could contribute to the spectral feature. More strenuous centrifugation than used in the present study would be required to obtain the smaller vesicles. It is likely that both processes, i.e., the presence of non-bilayer phase regions and the association of the lipid vesicles, contribute to the formation of the spectral peak.

The, in vivo, vesicles, which have been described in Hb SS disease [4,21], also occur, in vivo, in idiopathic autoimmune thrombocytopenic purpura [27], in hemolytic hereditary elliptocytosis, in Hb H disease, in hereditary spherocytosis [21], and in normal cells [28]. The vesicles appear to be present in significant numbers in sickle cell disease and could contribute to aPL generation. It is of interest that the systemic circulation of shed-vesicles may not be the significant indicator of vesicle importance since infusion of phospholipid vesicles into a localized thrombotic area can induce a systemic coagulation disorder, i.e., disseminated intravascular coagulation [29]. Platelet shed-vesicles, which have certain characteristics similar to sickle red cell shed-vesicles, do appear to contribute to the generation of aPL [30].

The precise non-bilayer phase, which the spectrum of the sickle RBC membrane reflects, is not clear. The spectral finding probably does not indicate an H_{11} non-bilayer phase, i.e., a three-dimensional membrane phase with two-dimensional ordering of the non-bilayer cylindrical structures, since the available non-bilayer forming lipid molecules in a RBC membrane would not be sufficient to

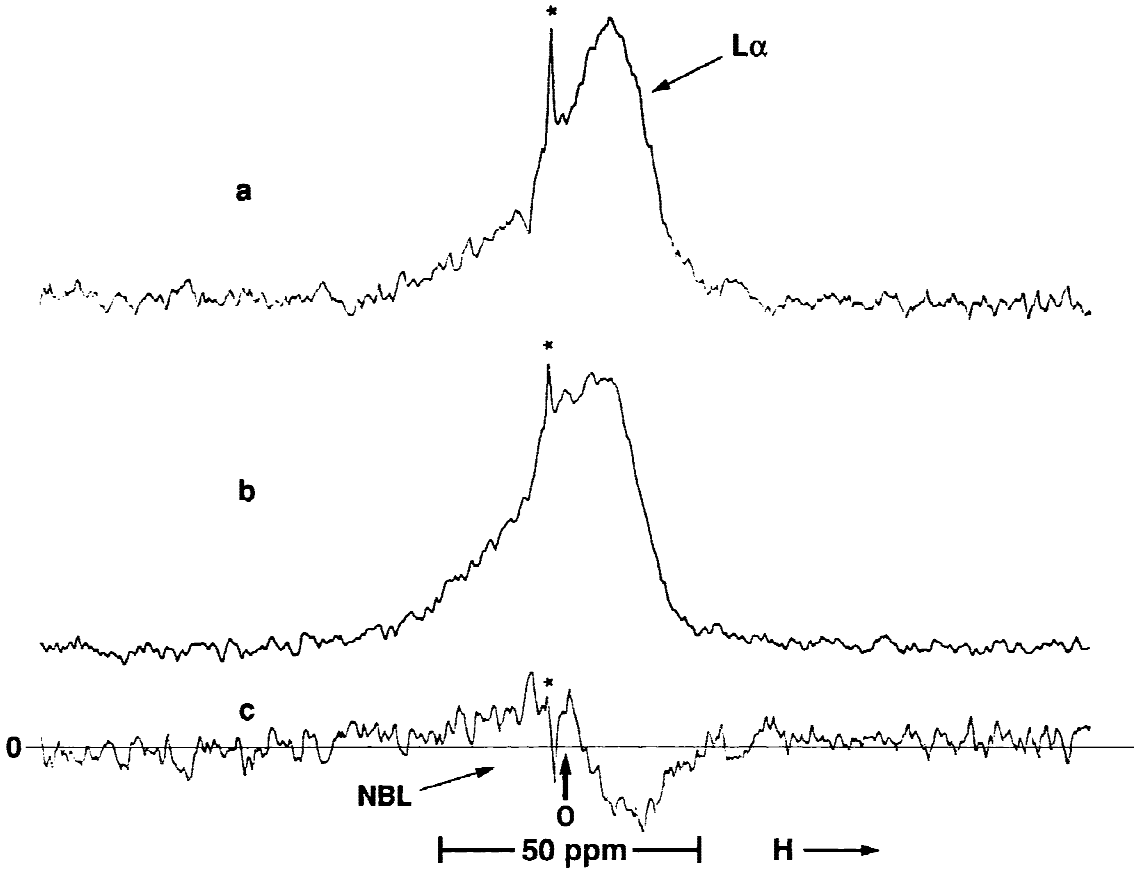

Fig. 3. Representative ^{31}P-NMR spectra of (a) normal red cell ghosts, (b) sickle red cell ghosts, and (c) the difference spectrum of [(b) − 1.31(a)]. The zero baseline for the difference spectrum is indicated in c. Lα = bilayer; NBL = nonbilayer; H = high field. *Ionic phosphate.

produce a stable H_{11} phase and there is an additional need to maintain membrane integrity as a barrier. Temporally transient inverted lipid cylinders or micelles in the membrane also would not produce the large spectral feature expected for an H_{11} lipid phase although their presence would cause an increase in the low field spectral intensity. To what extent the intramembranous particles described in sickle RBC membranes [31] are related to the inverted micellar structure associated with nonbilayer phase changes, i.e., intramembranous lipidic particles [32], is not known.

In conclusion, the ^{31}P-NMR spectral findings in these studies indicate that the membranes of the sickle red cell shed-vesicles and the membranes of sickle red cells show evidence of non-bilayer phases within the bilayer phase, probably in domains and in certain cell fractions. The non-bilayer phases in the vesicles could occur as a function of membrane compositional remodeling [33] or possibly in association with membrane deformation and fusion, which would occur during sickling of the cells [5]. The non-bilayer phases in the sickle red cells could be related to the type of cell, i.e., normal vs. diseased, as well as to compositional remodelling, membrane remodelling [33], or to membrane deformation and fusion that occur during sickling [5]. The phase changes in the vesicle and red cell membranes could contribute to the formation of antiphospholipid antibodies and, potentially, to the clotting abnormalities observed in sickle cell disease.

REFERENCES

1. Westerman MP, Cole ER, Wu K: The effects of spicules obtained from sickle red cells on clotting activity. Br J Haematol 56:557–562, 1984.
2. Franck PH, Bevers EM, Lubin BH, Comfurius P, Chin DT-Y, Opden Kamp JAF, Zwaal RFA, Van Deenen LLM, Roelofsen B: Uncoupling of the membrane skeleton from the lipid bilayer: The cause of accelerated phospholipid flip-flop leading to an enhanced procoagulant activity of sickled cells. J Clin Invest 75:183–190, 1985.
3. Choe HR, Schlegel HR, Rubin E, Williamson P, Westerman MP: Alteration of red cell organization in sickle cell anaemia. Br J Haematol 64:761–773, 1986.
4. Allan D, Limbrick AR, Thomas P, Westerman MP: Release of spectrin free spicules on reoxygenation of sickled erythrocytes. Nature 295: 612–613, 1982.
5. Zwall RFA, Comfurius P, Bevers EM: Mechanism and function of changes in membrane-phospholipid asymmetry in platelets and erythrocytes. Biochem Soc Trans 21:248–255, 1993.

6. Galli M, Bevers EM: Blood cell lipid asymmetry and antiphospholipid antibodies. Hemostasis 24:183–190, 1994.
7. Etzion Z, Tiffert T, Bookchin RM, Lew V: Effects of deoxygenation on active and passive Ca^{2+} transport and on the cytoplasmic Ca^{2+} levels of sickle cell anemia red cells. J Clin Invest 92:2489–2498, 1993.
8. Westerman MP, Puchulu E, Schlegel RA, Ahmend M, Williamson P: Chlortetracycline stained red cell inclusions in sickle cell disorders: An evaluation and correlation with clinical severity. J Lab Clin Med 124: 416–420, 1994.
9. Cullis PR, Hope MJ, de Kruijff B, Verkleij AJ, Tilcock CPS: In Kuo JF (ed): Phospholipids and Cellular Regulation. Boca Raton, FL: CRC Press, 1985, Chapter 1, pp 1–59.
10. Kucuk O, Lis LJ, Dey T, Mata R, Westerman MP, Yachnin S, Szostek R, Tracy D, Kauffman JW, Gage DA, Sweeley CC: The effects of cholesterol oxidation products in sickle and normal red blood cells membranes. Biochim Biophys Acta 1103:296–302, 1992.
11. Epand RM, Bottega R: Modulation of the phase transition behavior of phosphatidylethanolamine by cholesterol and oxysterols. Biochemistry 26:1820–1825, 1987.
12. Lis LJ, Kucuk O, Westerman MP, Tamura-Lis W, Quinn PJ, Cunningham BA, Collins JM, Wolfe DH: Modulation of phospholipid phase structures and transitions induced by oxysterols. Mol Cryst Liq Cryst 213:213–222, 1992.
13. Lubin B, Chiu D, Bastacky J, Roelofson B, van Deenan LLM: Abnormalities in membrane phospholipid organization in sickled erythrocytes. J Clin Invest 67:1643–1649, 1981.
14. Rauch J, Janoff AS: Phospholipid in the hexagonal H_{11} phase is immunogenic: Evidence for recognition of nonbilayer lipid phases in vivo. Proc Natl Acad Sci USA 87:4112–4114, 1990.
15. De Ceulaer K, Khamashta MA, Harris EN, Serjeant GR, Hughes GRV: Antiphospholipid antibodies in homozygous sickle cell disease. Ann Rheum Dis 51:671–72, 1992.
16. Kucuk O, Gilman-Sachs A, Beaman K, Lis LJ, Westerman MP: Antiphospholipid antibodies in sickle cell disease. Am J Hematol 42:380–383, 1993.
17. Francis Jr RB, Hebbel RP: In Embury SH, Hebbel RP, Mohandas N, Steinberg MH (eds): Sickle Cell Disease: Basic Principles and Clinical Practice. New York: Raven Press, 1994, pp 299–310.
18. McNeil HP, Chesterman CN, Krilis SA: Immunology and clinical importance of antiphospholipid antibodies. Adv Immunol 49:193–280, 1991.
19. Cullis PR, Hope MJ: Effects of fusogenic agent on membrane structure of erythrocyte ghosts and the mechanism of membrane fusion. Nature 271:672–674, 1978.
20. Allan D, Limbrick AR, Thomas P, Westerman MP: Microvesicles from sickle erthrcytes and their relation to irreversible sickling. Br J Haematol 47:383–390, 1981.
21. Wagner GM, Chiu D.T-Y, Yee MC, Lubin BH: Red cell vesiculation: A common membrane physiologic event. J Lab Clin Med 108:315–324, 1986.
22. Green GA, Kalra VK: Sickling-induced binding of immunoglobulin to sickle erythrocytes. Blood 71:636–639, 1988.
23. Kuypers FA, Lewis RA, Hua M, Schott MA, Discher D, Ernst JD, Lubin BH: Detection of altered membrane phospholipid asymmetry in subpopulations of human red blood cells using fluorescently labelled annexin V. Blood 87:1179–1187, 1996.
24. Siegel DP, Banschbach J, Alford D, Ellens H, Lis LJ, Quinn PJ, Yeagle PL, Bentz J: Physiological levels of diacylglycerols in phospholipid membranes induce membrane fusion and stabilize inverted phases. Biochemistry 28:3703–3709, 1989.
25. Allan D, Thomas P, Limbrick AR: The isolation and characterization of 60 nm vesicles (''Nanovesicles'') produced during ionophore A23187-induced budding of human erythrocytes. Biochem J 188:881–887, 1980.
26. Johnstone RM, Adam M, Hammond JR, Orv L, Turbide C: Vesicle formation during reticulocyte maturation. J Biol Chem 262:9412–9420, 1987.
27. Zucker-Franklin D, Karpatkin S: Red cell and platelet fragmentation in idiopathic autoimmune thrombocytopenic purpura. N Engl J Med 297: 517–523, 1977.
28. Dumaswala UJ, Greenwalt TJ: Human erythrocytes shed exocytic vesicles in vivo. Transfusion 24:490–492, 1984.
29. Taylor Jr FB, He SE, Chang ACK, Box J, Ferrel G, Lee D, Lockhart M, Peer G, Esmon CT: Infusion of phospholipid vesicles amplifies the local thrombotic response to TNF and anti-protein C into a consumptive response. Thromb Haemost 75:578, 1996.
30. Galli M, Bevers E: Inhibition of phospholipid-dependent coagulation reactions by ''antiphospholipid antibodies'': Possible modes of action. Lupus 3:223–228, 1994.
31. Lessin LS, Kurantsin-Mills J, Wallas C, Weems H: Membrane alterations in irreversibly sickled cells: Hemoglobin-membrane interaction. J Supramol Struct 9:537–554, 1978.
32. Verkleij AJ, Mombers C, Leumissen-Bijvelt J, Ververgaert PHJTR: Lipidic intramembranous particles. Nature 279:162–163, 1979.
33. Kirk GL, Gruner SM, Stein DL: A thermodyanic model of the lamellar to inverse hexagonal phase transition of lipid membrane-water systems. Biochemistry 23:1093–1102, 1984.